

**Ecotoxicity of nanomaterials in relation to the freshwater microalga
Raphidocelis subcapitata.**

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Abstract

Silver is the most frequently represented nanomaterial in available products, ranging across a variety of commercial and medical goods. Their main justification for inclusion in these products relates to their unique properties compared to both their bulk (larger particulate) and ionic equivalents. It is established that silver is highly toxic to a range of aquatic organisms, and despite its existence in low concentrations in the environment, concerns have been raised over products containing silver nanomaterials as a potential emerging pollutant. Within the EU the environmental risk assessment of chemicals is regulated by the Registration, Evaluation and Authorization of Chemicals (REACH) regulation, and toxicity testing deployed according to a series of tiered production/import thresholds. At the lowest regulatory production/import threshold of one tonne, the Organisation for Economic Co-operation and Development (OECD) 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test can be deployed to evaluate the toxicity to the aquatic environment via assessment of the impact of chemicals on algal species, over a 72 hour test duration. Due to the unique physico-chemico properties of nanomaterials, there is considerable uncertainty over the suitability of such test protocols for these materials. Thus, there may be a requirement for alternative test systems. In addition, the proliferation of the nanotechnology industry has created a diverse range of materials whose safety needs to be assessed. There is therefore an immediate need for rapid environmental risk assessment in order to keep pace with commercial activities. This thesis addresses such considerations in four stages. First, the OECD 201 test was applied to evaluate the toxicity of a representative silver nanoparticle (20-150 $\mu\text{g/l}$ Ag as NM300K, at 24-72 hours) and AgNO_3 (0.2-30 $\mu\text{g/l}$ Ag, at 24-72 hours), following dispersion in two different growth media, and the influence of different medium conditions such as humic acid (through the use of environmentally relevant concentrations of Suwannee River humic acid, [5 and 50 mg/l] and pH (6 and 8) on silver nanoparticle toxicity was investigated. In addition, a miniaturised version of the OECD 201 test was developed and compared to the standard test method, as a means to increase the rate and efficiency at which laboratories can generate toxicity data. Finally an alternative, short term sublethal photosynthetic endpoint was investigated in order to identify an alternative measure of toxicity to algae and inform the toxic mechanism of action of silver to algae, and correlations with the standard growth inhibition endpoint presented. It was found that the OECD 201 test was suitable to evaluate toxicity in NM300K and enabled identification of a 72 hour growth inhibition effective concentration (EC_{50}) of 54-130 $\mu\text{g/l}$ Ag. Of interest was that silver nanoparticle toxicity decreased with exposure duration and were affected by test medium composition. AgNO_3 toxicity was higher than NM300K (72 hour EC_{50} = 5-7 $\mu\text{g/l}$ Ag), but subject to the same time and media effects Humic acid decreased the toxicity of both forms of silver in a

concentration dependent manner, and pH 8 test medium increased toxicity relative to pH 6. The proposed miniaturised test was found to be as sensitive as the standard method (for NM300K) across a similar range of test conditions. Finally, the short-term photosynthetic sublethal endpoint indicated that silver may act in a similar way to known phototoxic substances, and by measuring this endpoint a high correlation was found with later 72 hour growth inhibition. Obtained data therefore demonstrate that the OECD 201 test can be used with *R. subcapitata* as a tool to assess nanoparticle toxicity, and that there are opportunities to increase the efficiency of testing via miniaturisation of the test system and the use of additional toxicity endpoints.

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"We take a handful of sand from the endless landscape of awareness around us and call that handful of sand the world."

— Robert M. Pirsig (1974), *Zen and the Art of Motorcycle Maintenance: An Inquiry into Values*

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Figure 3.4: Experimental growth rate of *R. subcapitata* in control cultures of OECD medium (black, humic acid free), and medium containing 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean toxicant free control growth and standard error of the mean (n_{control} = 24; n_{SRHA} = 12). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

Figure 3.5: Impact of NM300K [190 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* growth inhibition, in OECD medium. NM300K growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). Letters denote significant differences within each timepoint (Kruskal-Wallis; p < 0.05); bars not sharing the same letter were significantly different.

Figure 3.6: Impact of AgNO₃ [6 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* growth inhibition, in OECD medium. AgNO₃ growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different. A Kruskal-Wallis test at 24 hours found significant differences (p < 0.001) in growth inhibition between humic acid conditions, but a Mann-Whitney U test was unable to identify specific differences.

Figure 3.7: Daily pH measurements of control cultures (toxicant free) of *R. subcapitata* in unbuffered OECD medium (black circles, solid line), and OECD medium buffered (using 3.5 mM 3-(N-morpholino)propanesulfonic acid) to pH 6 (grey squares, dotted line) and pH 8 (white squares, dashed line). Data expressed as mean toxicant free control pH and standard error of the mean (error bars smaller than markers; n = 3).

Figure 3.8: Daily conductivity measurements of control cultures (toxicant free) of *R. subcapitata* in unbuffered OECD medium (black circles), and OECD medium buffered (using 3.5 mM 3-(N-morpholino)propanesulfonic acid) to pH 6 (grey squares) and pH 8 (white squares). Data expressed as mean toxicant free control pH and standard error of the mean (error bars smaller than markers; n = 3).

Figure 3.9: Experimental growth rate of *R. subcapitata* in control cultures of OECD medium (black, unbuffered), and medium buffered (using 3.5 mM 3-(N-morpholino)propanesulfonic acid) to pH 6 (grey) and pH 8 (white). Data expressed as mean toxicant free control growth and standard error of the mean ($n_{\text{control}} = 21$; $n_{\text{SRHA}} = 12$). Letters denote significant differences within each timepoint (One way ANOVA; $p < 0.05$); bars not sharing the same letter were significantly different.

Figure 3.10: Impact of pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on NM300K [190 $\mu\text{g/l}$ Ag] toxicity to *R. subcapitata*, in OECD medium. NM300K growth inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean ($n = 3$). Letters denote significant differences within each timepoint (One way ANOVA; $p < 0.05$); bars not sharing the same letter were significantly different.

Figure 3.11: Impact of pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on AgNO_3 [6 $\mu\text{g/l}$ Ag] toxicity to *R. subcapitata*, in OECD medium. NM300K growth inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean ($n = 1$, one experiment with three replicates). Letters denote significant differences within each timepoint (One way ANOVA; $p < 0.05$); bars not sharing the same letter were significantly different.

Figure 3.12: Impact of NM300K [20-60 $\mu\text{g/l}$ Ag] on *R. subcapitata* growth rate inhibition in JM, at 24 hours (black circles) 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean ($n = 3$).

Figure 3.13: Impact of AgNO_3 [1-30 $\mu\text{g/l}$ Ag] on *R. subcapitata* growth rate inhibition in JM, at 24 hours (black circles) 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean ($n = 1$, one experiment with three replicates).

Figure 3.14: Experimental growth rate of *R. subcapitata* in control cultures of JM (black, humic acid free), and medium containing 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid (SRHA). Data expressed as mean toxicant free control growth and standard error of the mean ($n_{\text{control}} = 22$; $n_{\text{SRHA}} = 12$). Letters denote significant differences within each timepoint (One way ANOVA; $p < 0.05$); bars not sharing the same letter were significantly different.

Figure 3.15: Impact of NM300K [80 $\mu\text{g/l}$ Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* growth inhibition, in JM. NM300K growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean ($n = 3$). Letters denote significant differences within each timepoint (One way ANOVA; $p < 0.05$); bars not sharing the same letter were significantly different.

Figure 3.16: Impact of AgNO₃ [6 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* growth inhibition, in OECD medium. AgNO₃ growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates). A Kruskal-Wallis test at each timepoint found significant differences (p < 0.001) in growth inhibition between humic acid conditions, but a Mann-Whitney U test was unable to identify specific differences.

Figure 3.17: Daily pH measurements of control cultures (toxicant free) of *R. subcapitata* in unbuffered JM (black circles, solid line), and JM buffered (using with 3.5 mM 3-(N-morpholino)propanesulfonic acid) to pH 6 (grey squares, dotted line) and pH 8 (white squares, dashed line). Data expressed as mean toxicant free control pH and standard error of the mean (error bars smaller than markers; n = 3).

Figure 3.18: Daily conductivity measurements of control cultures (toxicant free) of *R. subcapitata* in unbuffered OECD medium (black circles), and OECD medium buffered (using 3.5 mM 3-(N-morpholino)propanesulfonic acid) to pH 6 (grey squares) and pH 8 (white squares). Data expressed as mean toxicant free control pH and standard error of the mean (error bars smaller than markers; n = 3).

Figure 3.19: Experimental growth rate of *R. subcapitata* in control cultures of JM (black, unbuffered), and medium buffered (using 3.5 mM 3-(N-morpholino)propanesulfonic acid) to pH 6 (grey) and pH 8 (white). Data expressed as mean toxicant free control growth and standard error of the mean (n_{control} = 21; n_{SRHA} = 12). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

Figure 3.20: Impact of NM300K [80 µg/l Ag] and pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on *R. subcapitata* growth inhibition, in JM. NM300K growth inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

Figure 3.21: Impact of AgNO₃ [11 µg/l Ag] and pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on *R. subcapitata* growth inhibition, in JM. NM300K growth inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

Figure 4.1: Change *R. subcapitata* cell density (starting density 5 x 10⁴ cells/ml) in cultures grown in OECD medium, as determined by a) manual cell counts, b) optical density at 685 nm, c) *in vitro* Chl_a, d) *in vivo* Chl_a (Ex/Em: 435/685 nm). Data expressed as mean, and standard error of the mean (n = 3)

Figure 4.2: Maximum growth rate (d^{-1}) of *R. subcapitata* in OECD medium, measured by cell count, optical density (absorbance at 685 nm), *in vitro* Chl_a ($\mu g/l$) and *in vivo* Chl_a (Ex/Em: 435/685 nm) in flasks. Data expressed as mean growth rate and standard error of the mean ($n = 3$). Letters denote significant differences between each method of estimating cell density (One way ANOVA; $p < 0.05$); bars not sharing the same letter were significantly different.

Figure 4.3: Relationships between a series of cell density surrogate measures and cell counts: a) optical density vs cell counts; b) *in vitro* Chl_a vs cell counts; c) *in vivo* Chl_a vs cell counts (cells/ml); for *R. subcapitata* cultures grown in OECD medium. ($n = 30$)

Figure 4.4: Experimental growth rate of *R. subcapitata* in control cultures of OECD medium over 72 hours, estimated using different test systems; at 24 well plate *in vivo* Chl_a (black bars), flasks *in vitro* Chl_a (grey bars) and Flasks *in vivo* Chl_a (white bars). Dashed line represents minimum required 72 hour growth rate, according to OECD test guideline ($0.92 d^{-1}$). Data expressed as mean growth rate and standard error of the mean ($n = 24$). Letters denote significant differences between methods at each timepoint (One way ANOVA; $p < 0.05$); bars not sharing the same letter were significantly different.

Figure 4.5: Comparison between *R. subcapitata* control growth rate (μ) in OECD medium between the 24 well plate test and the OECD standard method (conducted in flasks, *in vivo* Chl_a extraction) after 24 hours (black circles), 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean toxicant free control growth rate of a single experimental condition and standard error of the mean (six replicates); $n = 39$. Solid line represents equal growth rate boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results. Dashed lines represent minimum required 72 hour growth rate, according to OECD test guidelines ($0.92 d^{-1}$).

Figure 4.6: Comparison between *R. subcapitata* control growth rate (μ) in OECD medium between the 24 well plate test and the OECD standard method (conducted in flasks, *in vitro* Chl_a extraction) after 24 hours (black circles), 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean toxicant free control growth rate of a single experimental condition and standard error of the mean (six replicates); $n = 39$. Solid line represents equal growth rate boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results. Dashed lines represent minimum required 72 hour growth rate, according to OECD test guidelines ($0.92 d^{-1}$).

Figure 4.7: Comparison between *R. subcapitata* control growth rate (μ) in OECD medium between from two methods of measuring growth in the same OECD standard test system (flasks; *in vivo* and *in vitro* Chl_a) after 24 hours (black circles), 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean toxicant free control growth rate of a single experimental condition and standard error of the mean (six replicates); $n = 42$. Solid line represents equal growth rate boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results. Dashed lines represent minimum required 72 hour growth rate, according to OECD test guidelines ($0.92 d^{-1}$).

Figure 4.8: Impact of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [0.2-100 µg/l] on *R. subcapitata* growth rate inhibition at 72 hours in OECD medium, in flasks/*in vivo* Chl_a (black circles), flasks/*in vitro* Chl_a (grey squares) and 24 well plates/*in vivo* Chl_a (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 1, one experiment with three replicates).

Figure 4.9: Impact of NM300K [30-150 µg/l] on *R. subcapitata* growth rate inhibition at 72 hours in OECD medium, in flasks/*in vivo* Chl_a (black circles), flasks/*in vitro* Chl_a (grey squares) and 24 well plates/*in vivo* Chl_a fluorescence (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 2).

Figure 4.10: Impact of AgNO₃ [0.2-20 µg/l] on *R. subcapitata* growth rate inhibition at 72 hours in OECD medium, in flasks/*in vivo* Chl_a (black circles), flasks/*in vitro* Chl_a (grey squares) and 24 well plates/*in vivo* Chl_a fluorescence (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 1, one experiment with three replicates).

Figure 4.11 *In vivo* Chl_a data for three replicate experiments with *R. subcapitata* (NM300K dose response; both controls and toxicant levels included) assessed via 24 well plate. A positive correlation was observed between measurements made in black and clear 24 plates ($r^2 = 0.98$; $p < 0.05$) (n = 189).

Figure 4.12: Experimental growth rate of *R. subcapitata* in control cultures of OECD medium over 72 hours in the presence and absence of Suwannee River humic acid (5 and 50 mg/l). Growth rate was estimated using different density surrogate methods/test systems; 24 well plate *in vivo* Chl_a (black bars), flasks *in vitro* Chl_a (grey bars) and flasks *in vivo* Chl_a (white bars). Data expressed as mean growth rate and standard error of the mean (n = 3). Letters denote significant differences between methods within each humic acid condition (One way ANOVA/Kruskal-Wallis; $p < 0.05$); bars not sharing the same letter were significantly different.

Figure 4.13: Impact of NM300K [190 µg/l Ag] on *R. subcapitata* 72 hour growth inhibition in the presence and absence of Suwannee River humic acid (SRHA; 5 and 50 mg/l). Growth inhibition was estimated using different density surrogate methods/test systems; 24 well plate *in vivo* Chl_a (black bars), flasks *in vitro* Chl_a (grey bars) and flasks *in vivo* Chl_a (white bars). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 2). Letters denote significant differences within each humic acid condition (Kruskal-Wallis; $p < 0.05$); bars not sharing the same letter were significantly different.

Figure 4.14: Impact of AgNO₃ [6 µg/l Ag] on *R. subcapitata* 72 hour growth inhibition in the presence and absence of Suwannee River humic acid (5 and 50 mg/l). Growth was estimated using different density surrogate methods/test systems; 24 well plate *in vivo* Chl_a (black bars), flasks *in vitro* Chl_a (grey bars) and flasks *in vivo* Chl_a (white bars). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates). Letters denote significant differences within each humic acid condition (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

Figure 4.15: Experimental growth rate of *R. subcapitata* in control cultures of OECD medium over 72 hours at a pH of 6 or 8. Growth rate was estimated using different density surrogate methods/test systems; 24 well plate *in vivo* Chl_a (black bars), flasks *in vitro* Chl_a (grey bars) and flasks *in vivo* Chl_a (white bars). Data expressed as mean growth rate and standard error of the mean (n = 3). Letters denote significant differences between methods within each pH condition (One way ANOVA/Kruskal-Wallis; p < 0.05); bars not sharing the same letter were significantly different.

Figure 4.16: Impact of NM300K [190 µg/l Ag] on *R. subcapitata* 72 hour growth inhibition at a pH of 6 or 8. Growth was estimated using different density surrogate methods/test systems; 24 well plate *in vivo* Chl_a (black bars), flasks *in vitro* Chl_a (grey bars) and flasks *in vivo* Chl_a (white bars). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). Letters denote significant differences within each pH condition (One way ANOVA/Kruskal-Wallis; p < 0.05); bars not sharing the same letter were significantly different. No significant differences were found between tests designs.

Figure 4.17: Impact of AgNO₃ [6 µg/l Ag] on *R. subcapitata* 72 hour growth inhibition at a pH of 6 or 8. Growth was estimated using different density surrogate methods/test systems; 24 well plate *in vivo* Chl_a (black bars), flasks *in vitro* Chl_a (grey bars) and flasks *in vivo* Chl_a (white bars). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). (n = 1, one experiment with three replicates). Letters denote significant differences within each pH condition (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

Figure 4.18: Comparison between *R. subcapitata* growth inhibition from 24 well plate test and the OECD standard method (conducted in flasks, *in vitro* Chl_a extraction) for AgNO₃ [6 µg/l] and NM300K [190 µg/l]. Growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. 0 mg/l (black circles), 5 mg/l (grey squares) and 50 mg/l (white triangles) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) of a single experimental condition (three replicates) and standard error of the mean. Correlation between flasks using *in vitro* Chl_a and 24 well plates using *in vivo* Chl_a (Pearson's product-moment correlation, n = 81 total observations, r² = 0.797, p < 0.001). Solid line represents equal growth inhibition boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results.

Figure 4.19: Comparison between *R. subcapitata* growth inhibition from 24 well plate test and the OECD standard method (conducted in flasks, *in vitro* Chl_a extraction) for AgNO₃ [6 µg/l] and NM300K [190 µg/l]. Growth inhibition was calculated relative to equivalent pH buffering (using 3.5mM 3-(N-morpholino)propanesulfonic acid), in toxicant free controls; i.e. unbuffered (black circles), pH 6 (grey squares) and pH 8 (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) of a single experimental condition (three replicates) and standard error of the mean. Correlation between flasks using *in vitro* Chl_a and 24 well plates using *in vivo* Chl_a (Pearson's product-moment correlation, n = 108 total observations, $r^2 = 0.810$, $p < 0.001$). Solid line represents equal growth inhibition boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results.

Figure 4.20: Comparison between *R. subcapitata* growth inhibition from 24 well plate test and the OECD standard method (conducted in flasks, *in vivo* Chl_a extraction) for AgNO₃ [6 µg/l] and NM300K [190 µg/l]. Growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. 0 mg/l (black circles), 5 mg/l (grey squares) and 50 mg/l (white triangles) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) of a single experimental condition (three replicates) and standard error of the mean. Correlation between flasks using *in vivo* Chl_a and 24 well plates using *in vivo* Chl_a (Pearson's product-moment correlation, n = 81 total observations, $r^2 = 0.840$, $p < 0.001$). Solid line represents equal growth inhibition boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results.

Figure 4.21: Comparison between *R. subcapitata* growth inhibition from 24 well plate test and the OECD standard method (conducted in flasks, *in vivo* Chl_a extraction) for AgNO₃ [6 µg/l] and NM300K [190 µg/l]. Growth inhibition was calculated relative to equivalent pH buffering (using 3.5mM 3-(N-morpholino)propanesulfonic acid), in toxicant free controls; i.e. unbuffered (black circles), pH 6 (grey squares) and pH 8 (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) of a single experimental condition (three replicates) and standard error of the mean. Correlation between flasks using *in vivo* Chl_a and 24 well plates using *in vivo* Chl_a (Pearson's product-moment correlation, n = 108 total observations, $r^2 = 0.882$, $p < 0.001$). Solid line represents equal growth inhibition boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results.

Figure 4.22: Comparison between *R. subcapitata* growth inhibition from two methods of measuring growth in the same OECD standard test system (flasks; *in vivo* and *in vitro* Chl_a) for AgNO₃ [6 µg/l] and NM300K [190 µg/l]. Growth inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. 0 mg/l (black circles), 5 mg/l (grey squares) and 50 mg/l (white triangles) Suwannee River humic acid. Data expressed as mean percentage growth inhibition (compared to toxicant free controls) of a single experimental condition (three replicates) and standard error of the mean. Correlation between flasks using *in vitro* Chl_a and flasks using *in vivo* Chl_a (Pearson's product-moment correlation, n = 81 total observations, $r^2 = 0.940$, $p < 0.001$). Solid line represents equal growth inhibition boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results.

Figure 4.23: Comparison between *R. subcapitata* growth inhibition from two methods of measuring growth in the same OECD standard test system (flasks; *in vivo* and *in vitro* Chl_a) for AgNO₃ [6 µg/l] and NM300K [190 µg/l]. Growth inhibition was calculated relative to equivalent pH buffering (using 3.5mM 3-(N-morpholino)propanesulfonic acid), in toxicant free controls; i.e. unbuffered (black circles), pH 6 (grey squares) and pH 8 (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free controls) of a single experimental condition (three replicates) and standard error of the mean. Correlation between flasks using *in vitro* Chl_a and flasks using *in vivo* Chl_a (Pearson's product-moment correlation, n = 108 total observations, $r^2 = 0.879$, $p < 0.001$). Solid line represents equal growth inhibition boundary between tests, and dotted lines represent the factor of five boundaries for differences between the test results.

Figure 5.1: Representation of photosynthetic light reactions (aka the Z scheme), located on the thylakoid membrane of the chloroplast. Left to right: A photon (light) excites a single electron in the P680 reaction centre of photosystem II (PSII) to an elevated energy level, where it is passed to pheophytin (the primary electron acceptor in the electron transport chain). From here the electron is transferred to the primary (Q_a), and then to the secondary plastoquinone acceptor (Q_b), where it remains. Simultaneously, the oxidised P680 is reduced by the enzymatic photolysis of water (H₂O) into oxygen (1/2O₂), protons (2H⁺) and electrons (e⁻). An additional photon excites another single electron, which proceeds along the same pathway as the first to Q_b. The now fully reduced Q_bH₂ transfers the electron pair through cytochrome b₆f complex and plastocyanin, where they act as the electron donors for the P700 reaction centre in photosystem I (PSI). From here the electrons are further excited by photons, and pass to a yet unidentified acceptor (analogous to pheophytin in PSII). The electrons pass through a series of iron sulphur proteins bound to the thylakoid membrane. The final electron acceptor is oxidised nicotinamide adenine dinucleotide phosphate (NADP⁺), where it is reduced to NADPH by ferredoxin-NADP⁺ reductase in the stroma of the chloroplast (adapted from Consalvey *et al.*, 2005).

Figure 5.2: Impact of DCMU [0-100 µg/l] on *R. subcapitata* photosynthetic efficiency (ΦPSII) over 30 minutes of exposure in OECD medium. Toxicant was added after a 5 minute acclimation period and effect on ΦPSII was measured for the remaining 25 minutes. Data are expressed as mean ΦPSII and standard error of the mean (n = 1, one experiment with three replicates).

Figure 5.3: Impact of $K_2Cr_2O_7$ [0-100 mg/l] on *R. subcapitata* photosynthetic efficiency ($\Phi PSII$) over 30 minutes of exposure in OECD medium. Toxicant was added after a 5 minute acclimation period and effect on $\Phi PSII$ was measured for the remaining 25. Data expressed as mean $\Phi PSII$ and standard error of the mean ($n = 1$, one experiment with three replicates).

Figure 5.4: Impact of $AgNO_3$ [0-500 $\mu g/l$ Ag] on *R. subcapitata* photosynthetic efficiency ($\Phi PSII$) over 30 minutes of exposure in OECD medium. Toxicant was added after a 5 minute acclimation period and effect on $\Phi PSII$ was measured for the remaining 25. Data expressed as mean $\Phi PSII$ and standard error of the mean ($n = 1$, one experiment with three replicates).

Figure 5.5: Impact of NM300K [0-1000 $\mu g/l$ Ag] on *R. subcapitata* photosynthetic efficiency ($\Phi PSII$) over 30 minutes of exposure in OECD medium. Toxicant was added after a 5 minute acclimation period and effect on $\Phi PSII$ was measured for the remaining 25. Data expressed as mean $\Phi PSII$ and standard error of the mean ($n = 3$).

Figure 5.6: Impact of DCMU [0.2-100 $\mu g/l$] on *R. subcapitata* photosynthetic efficiency ($\Phi PSII$) in OECD medium in flasks, at 4 hours (black circles) and 24 hours (grey squares). Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls) and standard error of the mean ($n = 1$, one experiment with three replicates).

Figure 5.7: Impact of $AgNO_3$ [0.2-20 $\mu g/l$ Ag] on *R. subcapitata* photosynthetic efficiency ($\Phi PSII$) in OECD medium in flasks, at 4 hours (black circles) and 24 hours (grey squares). Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls) and standard error of the mean ($n = 1$, one experiment with three replicates).

Figure 5.8: Impact of NM300K [30-150 $\mu g/l$ Ag] on *R. subcapitata* photosynthetic efficiency ($\Phi PSII$) in OECD medium in flasks, at 4 hours (black circles) and 24 hours (grey squares). Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls) and standard error of the mean ($n = 3$).

Figure 5.9: Impact of NM300K [190 $\mu g/l$ Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. NM300K photosynthetic inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean ($n = 3$). Letters denote significant differences within each timepoint (4 hours, Kruskal-Wallis; 24 hours, one way ANOVA; $p < 0.05$); bars not sharing the same letter were significantly different.

Figure 5.10: Impact of AgNO₃ [6 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. AgNO₃ photosynthetic inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

Figure 5.11: Impact of NM300K [190 µg/l Ag] and pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. NM300K photosynthetic inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 3). Letters denote significant differences within each timepoint (Kruskal-Wallis; p < 0.05); bars not sharing the same letter were significantly different

Figure 5.12: Impact of AgNO₃ [6 µg/l Ag] and pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. NM300K photosynthetic inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates). Letters denote significant differences within each timepoint (One way ANOVA; p < 0.05); bars not sharing the same letter were significantly different.

Figure 5.13: Comparison of data obtained from OECD 201 algal growth inhibition testing and photosynthetic efficiency for both NM300K and AgNO₃ toxicity to *R. subcapitata* in OECD medium, at 4 (black circles, solid line) and 24 (grey squares, dashed line) hours. Cells were exposed to Suwannee River humic acid (0, 5 and 50 mg/l) and pH modification (unbuffered, pH 6 and pH 8 using 3.5 mM 3-(N-morpholino)propanesulfonic acid), containing either no toxicant, AgNO₃ [6 µg/l Ag] or NM300K [190 µg/l Ag]. A positive correlation was observed between 72 hour *in vivo* growth rate and photosynthetic efficiency at 4 ($r^2 = 0.64$; p < 0.001) and 24 ($r^2 = 0.72$; p < 0.001) hours (n = 168).

Figure A3.1: Impact of potassium dichromate (K₂Cr₂O₇) [0.03-10 mg/l] on *R. subcapitata* growth rate inhibition in OECD medium, at 24 hours (black circles) 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 1, one experiment with three replicates).

Figure A4.1: Impact of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) [0.2-100 µg/l] on *R. subcapitata* growth rate inhibition in OECD medium, at 24 hours (black circles) 48 hours (grey squares) and 72 hours (white triangles). Data expressed as mean percentage growth inhibition (compared to toxicant free control) and standard error of the mean (n = 1, one experiment with three replicates).

Figure A6.1: Impact of AgNO₃ [0.2-20 µg/l Ag] on *R. subcapitata* photosynthetic efficiency (ΦPSII) in OECD medium in flasks, at 4 hours (black circles) and 24 hours (grey squares). Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates).

Figure A6.2: Impact of NM300K [30-150 µg/l Ag] on *R. subcapitata* photosynthetic efficiency (ΦPSII) in OECD medium in flasks, at 4 hours (black circles) and 24 hours (grey squares). Data expressed as mean percentage photosynthetic efficiency inhibition (compared to toxicant free controls) and standard error of the mean (n = 3).

Figure A6.3: Impact of NM300K [190 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. NM300K photosynthetic inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 3).

Figure A6.4: Impact of AgNO₃ [6 µg/l Ag] and Suwannee River humic acid (5 and 50 mg/l) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. AgNO₃ photosynthetic inhibition was calculated relative to equivalent concentrations of Suwannee River humic acid, in toxicant free controls; i.e. humic acid free (black), 5 mg/l (grey) and 50 mg/l (white) Suwannee River humic acid. Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates).

Figure A6.5: Impact of NM300K [190 µg/l Ag] and pH (6 and 8; buffered using 3.5 mM 3-(N-morpholino)propanesulfonic acid) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. NM300K photosynthetic inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 3).

Figure A6.6: Impact of AgNO_3 [$6 \mu\text{g/l Ag}$] and pH (6 and 8; buffered using 3.5mM 3-(N-morpholino)propanesulfonic acid) on *R. subcapitata* photosynthetic inhibition, after 4 and 24 hour exposure in OECD medium. NM300K photosynthetic inhibition was calculated relative to equivalent pH buffering, in toxicant free controls; i.e. unbuffered (black), pH 6 (grey) and pH 8 (white). Data expressed as mean percentage photosynthetic inhibition (compared to toxicant free controls) and standard error of the mean (n = 1, one experiment with three replicates).